J Mater Sci: Mater Med (2012) 23:171–179
DOI 10.1007/s10856-011-4512-3

Encapsulation of Huh-7 cells within alginate-poly(ethylene glycol) hybrid microspheres

Redouan Mahou · Nhu Mai Tran · Murielle Dufresne · Cécile Legallais · Christine Wandrey

Received: 28 October 2011 / Accepted: 30 November 2011 / Published online: 9 December 2011

Abstract Novel calcium alginate poly(ethylene glycol) hybrid microspheres (Ca-alg-PEG) were developed and evaluated as potentially suitable materials for cell microencapsulation. Grafting 5–13% of the backbone units of sodium alginate (Na-alg) with α-amine-ω-thiol PEG maintained the gelling capacity in presence of calcium ions, while thiol end groups allowed for preparing chemically crosslinked hydrogel via spontaneous disulfide bond formation. The combination of these two gelling mechanisms yielded Ca-alg-PEG. Human hepatocellular carcinoma cells (Huh-7) were encapsulated in Ca-alg-PEG and calcium alginate beads (Ca-alg), and cultured for 2 weeks under agitation conditions. Immediately after completion of the microencapsulation, the cell viability was 60% and similar in Ca-alg-PEG and Ca-alg. The proliferation of Huh-7 encapsulated in Ca-alg-PEG was slightly higher than in Ca-alg. Accelerated proliferation after 2 weeks was observed for the encapsulation in Ca-alg-PEG. The production of albumin confirmed the functionality of the encapsulated Huh-7 cells. The study confirms the suitability of Ca-alg-PEG and the one-step technology for cell microencapsulation.

R. Mahou · C. Wandrey (✉)
Institut d'Ingénierie Biologique et Institut des Sciences et Ingénierie Chimiques, Ecole Polytechnique Fédérale de Lausanne, EPFL-SV-IBI-LMRP, Station 15, 1015 Lausanne, Switzerland
e-mail: christine.wandrey@epfl.ch

N. M. Tran · M. Dufresne · C. Legallais
CNRS UMR 6600 Biomécanique et Bioingénierie, Université de Technologie de Compiègne, 60205 Compiègne, France

1 Introduction

Liver tissue engineering with three-dimensional (3D) biomaterials represents a promising approach for developing hepatic tissue, which mimics liver functions. Many other applications would benefit from this approach, ranging from organ supply to toxicology studies and even virology [1–3]. It has been shown that 3D culture offers suitable conditions to induce maturation in fetal hepatocytes, and to prolong hepatic functions of primary adult hepatocytes [4–8]. Among the different 3D systems that have been developed for this purpose, encapsulation of hepatic cells within hydrogel microspheres is thought to advantageously meet the requirements for sustained cell viability and functionality [9–13]. Indeed, the encapsulation of cells within hydrogels of spherical shape and suitable surface/volume ratio is representing an interesting alternative to fiber systems [14, 15] or sandwich cultures [16–18]. The microencapsulated cells can be perfused within bioreactors [19–23] with a significant improvement of mass transfer between the cells and the surrounding fluid. Nevertheless, progress in the field of cell microencapsulation has been hampered in particular due to difficulties to maintain cell viability, insufficient mechanical stability of the hydrogels, and biocompatibility issues. Several criteria must be considered when designing suitable hydrogel microspheres for cell encapsulation. Microspheres intended for cell encapsulation require sufficient mechanical resistance and durability to withstand the various forces during application. In addition, the process by which gelation occurs must support cell integrity and viability [24]. Care should also be taken in selecting a suitable preparation technology. Because cells are suspended in a precursor solution prior to the encapsulation process, the choice of precursors is limited to water-soluble components.

Moreover, precursor solutions must be buffered with appropriate osmolality to prevent cell lysis, and their rheological properties have to be carefully controlled to maintain cell viability and cell–cell contact during the encapsulation process. For example, mixing cells with highly viscous solutions can lead to significant decrease in cell viability because high shear stress can damage cell membranes [25].

Due to the abundance of sodium alginate (Na-alg), its favorable gelling properties in presence of divalent cations, and obvious biocompatibility [26, 27], alginate-based hydrogels remain the most frequently used materials for cell immobilization so far reported. Although there is no doubt about the advantageous properties of Na-alg, the success of using pure ionically crosslinked alginate gel networks is uncertain. Indeed, gradual dissolution of such "physical hydrogel" under physiological conditions remains an issue that needs to be solved. Moreover, frequently used additional coating or reinforcement with polycations requires multi-step processes and can have an unwanted negative impact on the biocompatibility. This was recently demonstrated using a human whole blood model to evaluate the inflammatory properties of polycation coated alginate microspheres [28]. As a conclusion from the state-of-the-art, promising materials should probably be free of polycations and producible by a simple technology consisting preferably of only one process step. Very recent studies report the modification of Na-alg with biomolecules [29, 30]. In this sense, the modification of Na-alg for subsequent or simultaneous reinforcement of ionically crosslinked network by covalent crosslinking has gained interest and it is being increasingly investigated [31–35].

Considering the requirements of efficient cell microencapsulation, novel calcium alginate poly(ethylene glycol) hybrid microspheres (Ca-alg-PEG) have been prepared by combining ionotropic gelation of PEG-grafted sodium alginate (Na-alg-PEG) and covalent crosslinking via disulfide bond formation. Under physiological conditions, a one-step extrusion process of Na-alg-PEG into a calcium chloride bath yielded spherical hydrogel of controllable size. Simultaneously, the more time-consuming covalent crosslinking proceeds spontaneously without additional reagent and reinforces the initially obtained microspheres. The suitability of Ca-alg-PEG for cell microencapsulation was investigated by encapsulating human hepatocellular carcinoma cells (Huh-7). Sustained viability, proliferation and metabolic activity of the encapsulated Huh-7 cells were confirmed in vitro. The conclusions thereof create the basis for novel approaches to prepare hybrid hydrogels for several applications in the field of cell microencapsulation.

2 Materials and methods

2.1 Materials

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium citrate, 3-(N-morpholino) propanesulfonic acid (MOPS), tris(2-carboxyethyl)phosphine) (TCEP), calcium chloride dihydrate ($CaCl_2.2H_2O$), and sodium chloride (NaCl) were purchased from Sigma (Sigma-Aldrich, Switzerland). Na-alg (HV Kelton, lot no. 46198 A) was obtained from Kelco (San Diego, CA, USA). The intrinsic viscosity [η] in 0.1 M NaCl at 20°C and the molar guluronic acid fraction F_G have been analyzed as $[\eta]_{0.1\ M\ NaCl} =$ 930 ml/g and $F_G = 0.41$. Unless otherwise mentioned, all reagents were analytical grade and were used without further purification. The synthesis of Na-alg-PEG was carried out as described elsewhere [36]. Briefly, Na-alg has been modified employing aqueous carbodiimide chemistry to covalently graft α-amine-ω-thiol PEG onto the carboxylate groups of the Na-alg backbone. Purification of Na-alg-PEG was achieved by 4 days dialysis against distilled water. The degree of grafting, which refers to the percentage of reacted carboxylate groups, was determined by ^{1}H-NMR. The same batch of Na-alg was used to prepare Ca-alg beads and to synthesize Na-alg-PEG.

2.2 Preparation of hydrogels

The gelling capability of Na-alg-PEG following three different approaches was investigated. Spherical physical hydrogels were prepared by ionotropic gelation of Na-alg-PEG in presence of calcium ions. The solution was prepared with 2.2 wt% Na-alg-PEG in MOPS stock solution (10 mM MOPS + 0.45% NaCl, pH = 7.4) and extruded directly after complete dissolution into a gelation bath (150 mM $CaCl_2$ in 10 mM MOPS, pH = 7.4). After 10 min gelation, the spherical physical hydrogels were collected by filtration, washed twice with MOPS stock solution, and stored at 4°C in the washing solution.

The preparation of chemically crosslinking hydrogel was achieved via spontaneous disulfide bond formation. In practice, Na-alg-PEG was dissolved in MOPS stock solution with a final concentration of 5 wt%. The solution was vortexed for about 2 min, and the glass vial holding the solution was placed at 37°C under air atmosphere for 72 h.

Hybrid microspheres resulted from the combination of both gelling mechanisms, the fast ionotropic gelation and the slow formation of disulfide bonds. This was achieved by 72 h incubation in MOPS at 37°C subsequent to the extrusion into the $CaCl_2$ bath. Spherical physical hydrogels, chemically crosslinked hydrogels, and hybrid

microspheres will subsequently be designated as PH, CH, and Ca-alg-PEG, respectively.

2.3 Cell model

Huh-7 cells were obtained from Pr. Duverlie G. (Virology Laboratory, University Hospital, Amiens, France). Cell monolayers were cultured at 37°C under an atmosphere of 95% air and 5% CO_2 in high glucose Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Institute of Technology Jacques Boy, France). The medium was changed three times per week. The cells were cultured to confluence before microencapsulation and were used from passage 5–10.

2.4 Cell microencapsulation

All components used for cell microencapsulation were dissolved in HEPES solution (10 mM, pH = 7.4). Considering the humidity of the Na-alg powder, the solution was prepared with a concentration of 1.5 wt%. The solution was shaken overnight at 4°C for complete dissolution, sterile filtrated (0.2 μm), and stored at 4°C until use. Na-alg-PEG solution was freshly prepared 2 h before the microencapsulation process to avoid the formation of disulfide bonds prior to encapsulation. Na-alg-PEG with a degree grafting of 5% was used in this study. The solution was prepared with a concentration of 2.2 wt%, and sterilized by filtration (0.2 μm) before use. The gelation bath was prepared by dissolving 154 mM NaCl and 115 mM $CaCl_2$ in HEPES solution and sterilization by autoclaving. Huh-7 cells were removed from T-flasks following trypsin incubation (Trypsin EDTA in PBS, Institute of Technology Jacques Boy, France), washed with DMEM, counted, and centrifuged (1,000 rpm, 5 min). The supernatant was withdrawn, and the Na-alg stock solution (1.5 wt% in HEPES) was added to the cell pellet to a final concentration of 5×10^5 cells/ml of solution. The suspension of cells in Na-alg was gently homogenized by using a pipette. 10 ml of the homogeneous suspension was extruded into 100 ml of the gelation bath employing a coaxial air-flow droplet generator [37]. The droplets were allowed to gel for 10 min. The microspheres were collected by filtration, washed twice with DMEM, and finally cultured in DMEM high glucose supplemented with 10% FBS. An identical protocol was followed to encapsulate Huh-7 cells within Ca-alg-PEG microspheres. The sterile solution of Na-alg-PEG (2.2 wt% in HEPES) was added to cell pellet (5×10^5 cells/ml), homogenized and extruded. The cells encapsulated in either Ca-alg-PEG or Ca-alg were transferred to tissue culture flask 25 cm^2 (Grenier Bio-one, France), maintained at 37°C under an atmosphere of 95% air and 5% CO_2, and cultured in hydrodynamic condition on a rotary plate.

2.5 Cell viability

Cell viability was quantified using the lactate dehydrogenase (LDH) assay (Promega, France). The assay monitors the conversion of nicotinamide adenine dinucleotide phosphate (NADH) into nicotinamide adenine dinucleotide (NAD +) by LDH. The LDH released into the extracellular medium by damaged cells was expressed as percentage of the total LDH activity in the cells (% LDH released = extracellular LDH/(extracellular LDH + intracellular LDH)). Encapsulated cells were cultured in DMEM high glucose supplemented with 10% FBS. The culture medium was changed at days 0, 2, 6, and 13. After 24 h, 50 μl of culture media were withdrawn from the wells containing the cells within microspheres and placed in another 96-well plate. Fifty microlitres of the reaction mixture (LDH Kit) were added to each well. The plate was then incubated for 30 min at room temperature in the dark. The absorbance of the samples was monitored at 490 nm UV/vis spectroscopy (SPECTRAFLUO Plus, TECAN, Switzerland. To calculate the relative extent of cell death in the microspheres, 1% Triton X-100 was added in a well containing the encapsulated cells to lyse all cells in the microspheres at days 1, 3, and 7. The total amount of LDH released was then measured. The test was performed in triplicate ($n = 3$).

2.6 Cell proliferation

The proliferation of encapsulated Huh-7 cells was quantified by the Alamar blue assay (Invitrogen, France). The assay measures the redox capacity of cells due to the production of metabolites as a result of cell growth. At days 1, 3, 7 and 14 of culture, 5 ml of 10% (v/v) Alamar blue dye in DMEM was added to each culture flask. After 1 h incubation (37°C, 5% CO_2), 100 μl of supernatant was withdrawn and transferred to 96-well plates. The fluorescence was measured by using a spectrophotometer (SPECTRAFLUO Plus, TECAN, Switzerland) 560 nm excitation wavelength/590 nm emission wavelength. The test was performed in triplicate ($n = 3$).

2.7 Albumin production

At days 1, 2, 3, 7 and 14, samples of the cell culture medium were withdrawn and frozen for subsequent analysis of the albumin concentration. A sandwich enzyme-linked assay determined the albumin concentration as previously described [12]. Briefly, 96-well plates were coated with goat antibody against human albumin (Cappel

Laboratories, Cochrainville, France) overnight at 4°C. The plates were washed, and non-specific interactions were subsequently blocked. Samples and standards of human albumin were then placed in wells and incubated for 90 min. The wells were then washed and incubated with an antibody against human albumin produced in goat and horseradish peroxidase (HRP) conjugated (Cappel Laboratories, Cochrainville, France). The wells were incubated after washing, adding H_2O_2 and substrate o-phenylenediamine dihydrochloride (OPD) for the HRP. The reaction was stopped by addition of 2 N sulfuric acid (H_2SO_4). The absorbance was measured at 490 nm by spectrophotometry.

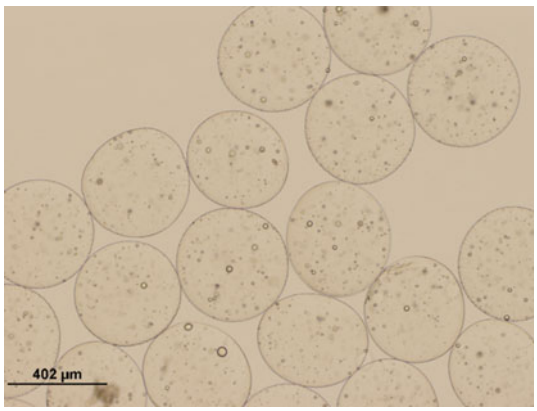

Fig. 2 Physical hydrogel (PH) obtained by ionotropic gelation

3 Results

3.1 Gelation of Na-alg-PEG

The gelling capability of Na-alg-PEG following three different pathways is schematically presented in Fig. 1. First, the remaining ability of Na-alg-PEG to form hydrogels by ionotropic gelation in presence of calcium ions was confirmed. Upon extrusion of a Na-alg-PEG solution into a receiving bath containing calcium ions, microspheres of uniform size, designated here as "physical hydrogel (PH)" were obtained (Fig. 2). Pegylation of up to 13% of chain units did not affect the ionotropic gelling capacity of Na-alg-PEG.

Secondly, hydrogel formation by only chemical crosslinking via disulfide bonds was confirmed when leaving a solution of Na-alg-PEG at 37°C in a glass vial for 72 h. The "chemically crosslinked hydrogel (CH)" was obtained with the shape of the vial holding the solution as shown in Fig. 3. Such CH is not obtainable using unmodified Na-alg. The reversibility of the resulting CH hydrogel was corroborated. Adding a solution of a reducing agent, in this study TCEP, cleaved the disulfide bonds and led to the liquefaction of the CH hydrogel (Fig. 3).

Combining the two mechanisms, fast ionotropic gelation and slow reinforcement by chemical crosslinking, yielded Ca-alg-PEG. After 72 h, chemical crosslinking was confirmed by incubation of the Ca-alg-PEG microspheres in

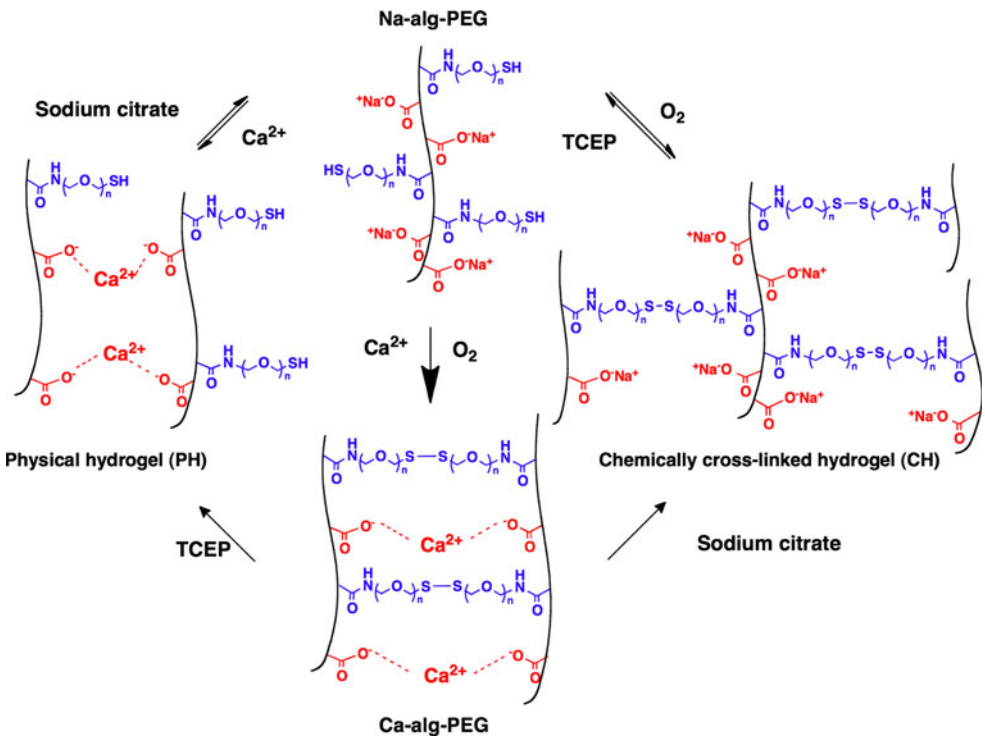

Fig. 1 Gelling pathways of Na-alg-PEG

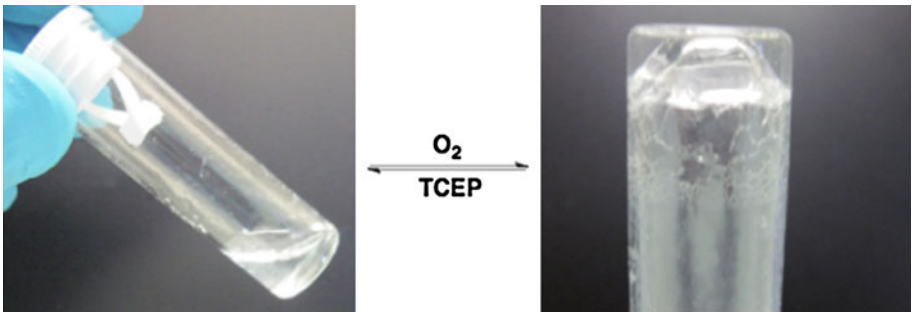

Fig. 3 Reversible chemically crosslinked hydrogel (CH) obtained via disulfide bond formation under air atmosphere

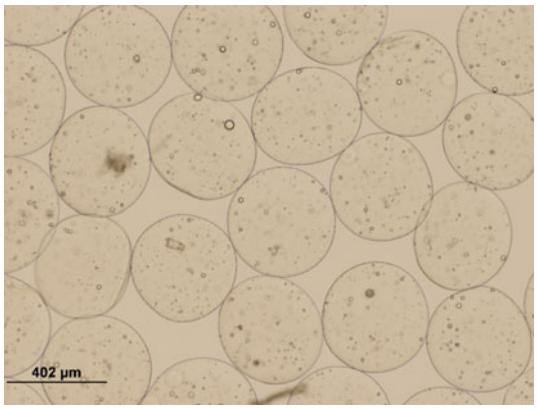

Fig. 4 Ca-alg-PEG remained stable and spherical after 24 h incubation in sodium citrate

sodium citrate solution. As visible in Fig. 4, Ca-alg-PEG kept the spherical shape. Neither deformation nor dissolution was observed.

3.2 Cell microencapsulation

The feasibility of the cell entrapment and the suitability of Ca-alg-PEG for cell microencapsulation were investigated. A one-step extrusion process under physiological conditions yielded Huh-7 cells encapsulated within Ca-alg and within the novel Ca-alg-PEG (Fig. 5). No free cells were identified in the gelation bath after separation of the microspheres. The average diameters of Ca-alg and Ca-alg-PEG were 400 and 700 µm. The study was limited to 2 weeks. During this period, the viability of the encapsulated cells, proliferation, and the production of albumin were investigated.

3.3 Cell viability

Figure 6 presents the results of the viability analyses toward the LDH assay. The viability of Huh-7 hepatocytes was first determined immediately after completion of the microencapsulation as in the range of 55–60%. It increased with time to reach 65–80% at 7 or 14 days. The cell viability was similar for both materials Ca-alg-PEG and Ca-alg at all time points of culture.

3.4 Cell proliferation

The Alamar blue assay allowed for assessing the cell proliferation within Ca-alg and Ca-alg-PEG. Figure 7 shows the fluorescence intensity over 14 days, which indicates slight differences between the two types of hydrogel, the cell growth being slightly superior in Ca-alg-PEG compared to the control. Moreover, the proliferation seemed to reach a maximum after 7 days in Ca-alg, while it continued for Ca-alg-PEG up to 2 weeks.

3.5 Albumin production

The specific functional activity of encapsulated Huh-7 cells in both types of microspheres was investigated by quantifying the albumin production in the supernatant. Figure 8 shows the amount of albumin released within 24 h normalized to unit of cell metabolic activity. The production of albumin was similar for both hydrogels.

4 Discussion

Functionalization of the Na-alg carboxylate groups with heterobifunctional PEG (α-amine-ω-thiol PEG) was achieved via carbodiimide chemistry [36]. The extrusion of Na-alg-PEG into calcium ions yielded spherical PH. This reaction confirms that the conjugation of Na-alg with the PEG pendant side chain did not affect the ionotropic gelling capability of these molecules. However, no spherical hydrogel was obtained if the degree of grafting exceeded 13%, i.e., if more than every eighth backbone unit was modified (data not shown). It is suggested that the amount of remaining free carboxylate groups is then insufficient to form enough Ca-ionic bridges necessary for a stable PH. The Na-alg-PEG molecules utilized in this study had a degree of grafting of 5% $\pm$ 0.4, which was sufficient to form a stable PH but also a stable CH.

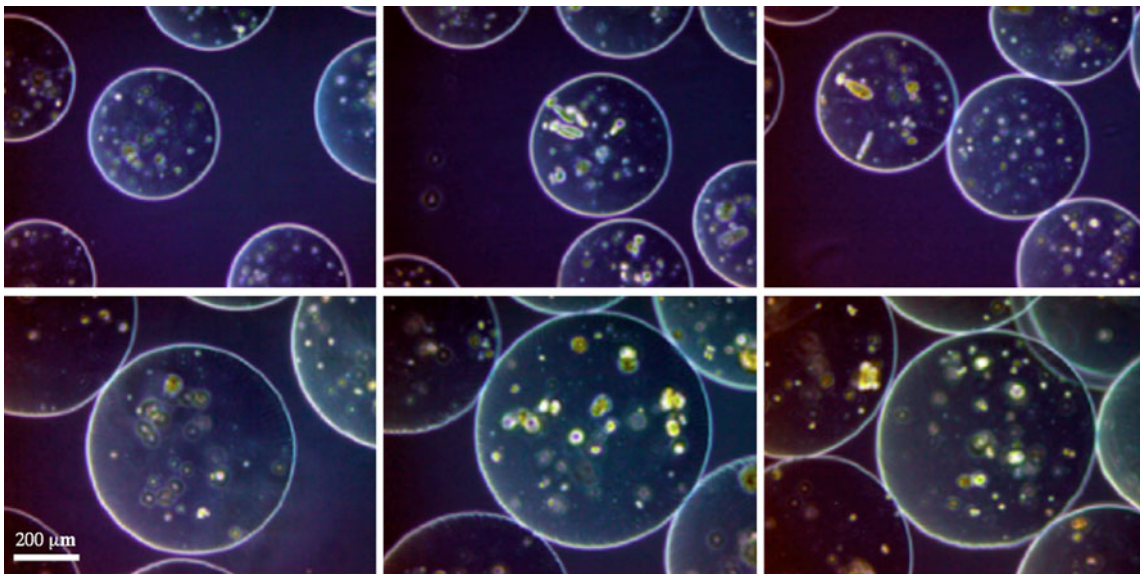

Fig. 5 Microphotographs of encapsulated Huh-7 cells within (top) Ca-alg and (bottom) Ca-alg-PEG. Microphotographs were taken (from *left* to *right*) at days 1, 7 and 14 applying the same magnification

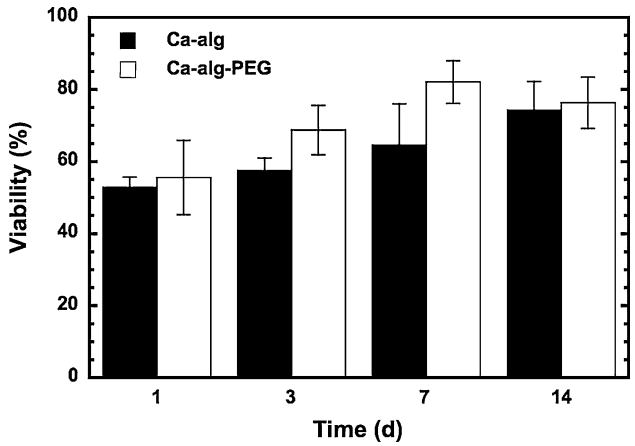

Fig. 6 The viability of encapsulated Huh-7 cells determined by the LDH assay. Values were normalized to the total number of cells

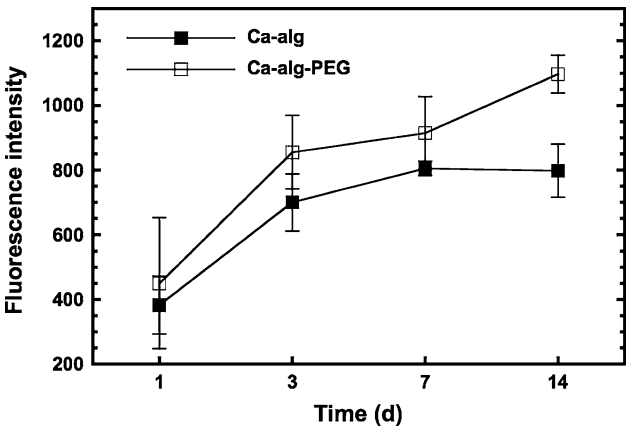

Fig. 7 Proliferation of encapsulated Huh-7 cells over 2 weeks evaluated by the Alamar *blue* assay

The approach of forming hydrogels via disulfide bond formation was reported for cell microencapsulation, drug delivery, and controlled protein release [38–40]. However, to our knowledge, the preparation of spherical alginate-based hydrogels with diameters in the micrometer range via disulfide bond formation was not reported so far.

Because of the spontaneous character of the oxidation reaction, the preparation of the CH was achievable under air atmosphere without adding any extra reagent. The kinetics of this reaction can be influenced by factors such as temperature, concentration, and oxygen concentration. So far, the time-consuming reaction (72 h) cannot be considered as a drawback. The spherical shape of hydrogel is assured by the initial fast ionotropic gelation of Na-alg-PEG after contact with Ca^{2+} ions. The advantage is that the reinforcement is achieved here without addition of polycations and without any other layering procedure.

Ca-alg-PEG was obtained by combining the two gelling mechanisms. The integrity and the sphericity of Ca-alg-PEG were maintained after 24 h incubation in sodium citrate used as calcium chelating agent. Our previous studies have shown that 10 min incubation in sodium citrate solution was sufficient to liquefy the hydrogel network prepared from unmodified Na-alg [35]. This observation confirms that efficient cross-linking via disulfide bond formation was achieved. Such crosslinking will certainly improve the durability of the Ca-alg-PEG if long-term uses in bioreactors or in vivo applications are intended.

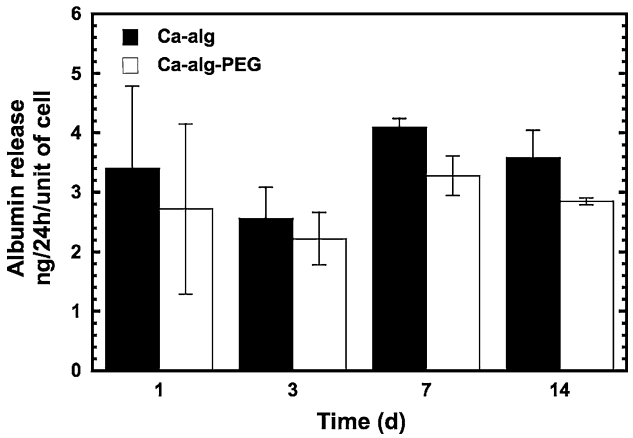

Fig. 8 Production of albumin by Huh-7 cells encapsulated within Ca-alg and Ca-alg-PEG

Huh-7 cells were encapsulated within Ca-alg and Ca-alg-PEG in a one-step extrusion process under physiological conditions (pH, temperature). For both hydrogels, the average diameter was tunable modifying the encapsulation conditions such as syringe diameter, extrusion rate, and/or airflow. The final average diameter was different when comparing both systems despite an identical microencapsulation protocol was followed. The pegylation of alginate led to improvement of the solubility of the polymer and consequently a reduction of the viscosity of the resulting solution. The extrusion of lower viscosity solutions yielded larger droplets, and thus, a larger average diameter upon gelation. Moreover, the PEG network could act as a spacer between alginate backbones. It therefore could reduce shrinkage of the droplets upon gelation, reported for alginate hydrogels [41]. This hydrogel shrinkage may create a mechanical stress on cells which can influence their viability during the microencapsulation process. In addition, the high degree of swelling of the hydrophilic PEG network could also have an impact on the final diameter of the microspheres. Nevertheless, both hydrogel types exhibit a stable size over time.

Cell viability was about 60% at day 1. This reduced viability was most likely due to the cell handling and/or encapsulation procedure, and not to the nature of the hybrid cross-linking. No significant difference was detected between Ca-alg and Ca-alg-PEG, suggesting that the formation of the PEG links via disulfide bonds did not negatively affect the cell survival. This finding was confirmed when studying the viability of encapsulated Huh-7 hepatocytes within Ca-alg-PEG over time. The viability did not decrease during the 3 days post microencapsulation, which was the required reaction time to complete the chemical PEG network, and improved until 14 days of incubation to reach around 75%. In other words, acceptable cell viability was obtained regardless the nature of the hydrogel material used.

Huh-7 cells encapsulated within Ca-alg-PEG continued proliferation up to 14 days, suggesting no detrimental effect of the encapsulation procedure on the ability of cells to proliferate. Ca-alg-PEG thus offered appropriate environment for cell proliferation, slightly better than Ca-alg. Cells encapsulated in Ca-alg-PEG form multicellular structures and spheroid aggregates (cluster). The size of these aggregates increased over 7 days of culture indicating cell proliferation, and then stabilized until 14 days.

Besides the survival and proliferation, albumin production by encapsulated Huh-7 cells continued, similar secretion of albumin within Ca-alg-PEG and Ca-alg was observed and. This production remained almost constant over time, up to 2 weeks.

The hydrogel chemically crosslinked with PEG seems to respond to limitations of alginate used alone for cell microencapsulation. The mechanical robustness of Ca-alg-PEG allows a physical protection for beads against shear stress as it is observed in fluidized bed bioartificial livers. In this bioreactor, plasma from the patient would be perfused through a column hosting encapsulated hepatocytes. The stable porosity over time of the hydrogel material could be a guarantee for an efficient immunoisolation [23]. This immune property is also required during long-term transplantation of encapsulated xenogenic cells or tissues.

5 Conclusions

With the aim to extend the materials basis for cell microencapsulation, we prepared novel hybrid microspheres from modified alginate molecules and studied the suitability of these microspheres for cell microencapsulation. The goal to obtain stable microspheres in a one-step process and without incorporation of polycations was achieved. It was found that Na-alg-PEG maintained the gelling capacity in presence of divalent cations, while the free thiol end groups allowed for simultaneous chemical crosslinking. This chemical crosslinking offers higher stability and durability upon exposition of the hydrogel to a chelating agent.

Overall, cell viability, proliferation and albumin secretion confirmed the suitability of Ca-alg-PEG for the microencapsulation of Huh-7 cells. The results were similar as for Ca-alg, which was included in the study for comparison and due to its known good biocompatibility. Remaining open questions concerning the durability of Ca-alg-PEG in long-term culture, mechanical resistance to shear stress in a bioreactor, and the suitability to encapsulate other cells are under investigation.

Acknowledgments The research was supported by the Swiss National Science Foundation (Grants 205320_130572/1 and 205321_116397/1)

References

1. Bierwolf J, Lutgehetmann M, Feng K, Erbes J, Deichmann S, Toronyi E, Stieglitz C, Nashan B, Ma PX, Pollok JM. Primary rat hepatocyte culture on 3D nanofibrous polymer scaffolds for toxicology and pharmaceutical research. Biotechnol Bioeng. 2011;108:141–50.
2. Sung JH, Kam C, Schuler ML. A microfluidic device for a pharmacokinetic-pharmacodynamic (PK-PD) model on a chip. Lab Chip. 2010;10:446–55.
3. Sainz B, Tencate V, Uprichard SL. Three-dimensional Huh7 cell culture system for the study of Hepatitis C virus infection. Virol J. 2009;6:103.
4. Hanada S, Kayano H, Jiang J, Kojima N, Miyajima A, Sakoda A, Sakai Y. Enhanced in vitro maturation of subcultivated fetal human hepatocytes in three dimensional culture using poly-L-lactic acid scaffolds in the presence of oncostatin M. Int J Artif Organs. 2003;26:943–51.
5. Iwahori T, Matsuura T, Maehashi H, Sugo K, Saito M, Hosokawa M, Chiba K, Suzuki T. CYP3A4 inducible model for in vitro analysis of human drug metabolism using a bioartificial liver. Hepatology. 2003;37:665–73.
6. Lin RZ, Chang HY. Recent advances in three-dimensional multicellular spheroid culture for biomedical research. Biotechnology. 2008;3:1172–84.
7. Miki T, Ring A, Gerlach JC. Three-dimensional dynamic perfusion culture conditions promote hepatic differentiation of human embryonic stem cells. Tissue Eng Part C Methods. 2011;17:557–68.
8. Prot JM, Videau O, Brochot C, Legallais C, Bénech H, Leclerc E. A cocktail of metabolic probes demonstrates the relevance of primary human hepatocyte culture in a microfluidic biochip for pharmaceutical drug screening. Int J Pharm. 2011;408:67–75.
9. Selden C, Shariat A, McCloskey P, Ryder T, Roberts E, Hodgson H. Three-dimensional in vitro cell culture leads to a marked upregulation of cell function in human hepatocyte cell lines-an important tool for the development of a bioartificial liver machine. Ann New York Acad Sci. 1999;875:353–863.
10. Khalil M, Shariat-Panahi A, Tootle R, Ryder T, McCloskey P, Roberts E, Hodgson H, Selden C. Human hepatocyte cell lines proliferating as cohesive spheroid colonies in alginate markedly upregulate both synthetic and detoxificatory liver function. J Hepatol. 2001;34:68–77.
11. Gao Y, Jiang HC, Xu J, Pan SH, Li YD. Microencapsulating hepatocytes. Transplant Proc. 2005;37:4589–93.
12. Kinasiewicz A, Gautier A, Lewinska D, Smietanka A, Legallais C, Werynski A. Three-dimensional growth of human hepatoma C3A cells within alginate beads for fluidized bioartificial liver. Int J Artif Organs. 2008;31:340–7.
13. Lan SF, Safiejko-Mrocka B, Starly B. Long-term cultivation of HepG2 liver cells encapsulated in alginate hydrogels: A study of cell viability, morphology and drug metabolism. Toxicol In Vitro. 2010;24:1314–23.
14. De Bartolo L, Morelli S, Lopez LC, Giorno L, Campana C, Salerno S, Rende M, Favia P, Detomaso L, Gristina R, Agostino R, Drioli E. Biotransformation and liver-specific functions of human hepatocytes in culture on RGD-immobilized plasma-processed membranes. Biomaterials. 2005;26:4432–41.
15. Seo SJ, Akaike T, Choi YJ, Shirakawa M, Kang IK, Cho CS. Alginate microcapsules prepared with xyloglucan as a synthetic extracellular matrix for hepatocyte attachment. Biomaterials. 2005;26:3607–15.
16. Berthiaume F, Moghe PV, Toner M, Yarmush ML. Effect of extracellular matrix topology on cell structure, function, and physiological responsiveness: hepatocytes cultured in a sandwich configuration. Faseb J. 1996;10:1471–84.
17. Schmitmeier S, Langsch A, Jasmund I, Bader A. Development and characterization of a small scale bioreactor based on a bioartificial hepatic culture model for predictive pharmacological in vitro screenings. Biotechnol Bioeng. 2006;95:1198–206.
18. Ohashi K, Yokoyama T, Yamato M, Kuge H, Kanehiro H, Tsutsumi M, Amanuma T, Iwata H, Yang J, Okano T, Nakajima Y. Engineering functional two- and three-dimensional liver systems in vivo using hepatic tissue sheets. Nat Med. 2007;13:880–5.
19. David B, Dufresne M, Nagel MD, Legallais C. In vitro assessment of encapsulated C3A hepatocytes functions in a fluidised bed bioreactor. Biotechnol Prog. 2004;20:1204–12.
20. Coward SM, Selden C, Mantalaris A, Hodgson HJ. Proliferation rates of HepG2 cells encapsulated in alginate are increased in a microgravity environment compared with static cultures. Artif Organs. 2005;29:152–8.
21. Coward SM, Legallais C, David B, Thomas M, Foo Y, Mavri-Damelin D, Hodgson HJ, Selden C. Alginate-encapsulated HepG2 cells in a fluidized bed bioreactor maintain function in human liver failure plasma. Artif Organs. 2009;33:1117–26.
22. Miranda JP, Rodrigues A, Tostoes RM, Leite S, Zimmerman H, Carrondo MJ, Alves PM. Extending hepatocyte functionality for drug-testing applications using high-viscosity alginate-encapsulated three-dimensional cultures in bioreactors. Tissue Eng Part C Methods. 2010;16:1223–32.
23. Gautier A, Carpentier B, Dufresne M, Vu Dinh Q, Paullier P, Legallais C. Impact of alginate type and bead diameter on mass transfers and the metabolic activities of encapsulated C3A cells in bioartificial liver applications. Eur Cells Mater. 2011;21:94–106.
24. Nicodemus GD, Bryant SJ. Cell encapsulation in biodegradable hydrogels for tissue engineering applications. Tissue Eng. Part B. 2008;14:149–65.
25. Schmidt JJ, Rowley J, Kong HJ. Hydrogels used for cell-based drug delivery. J Biomed Mater Res A. 2008;87:1113–22.
26. Santos E, Zarate J, Orive G, Hernández RM, Pedraz JL. Biomaterials for cell microencapsulation. Adv Exp Med Biol. 2010;670:5–21.
27. Hernández RM, Orive G, Murara A, Pedraz JL. Microcapsules and microcarriers for in situ cell delivery. Adv Drug Delivery Rev. 2010;62:711–30.
28. Rokstad AM, Brekke OL, Steinkjer B, Ryan L, Kolláriková G, Strand BL, Lacik I, Espevik T, Mollnes TE. Alginate microbeads are complement-compatible in contrast to polycation containing microcapsules revealed by a human whole blood model. Acta Biomater. 2011;7:2566–78.
29. Yang JS, Xie YJ, He W. Research progress on chemical modification of alginate: A review. Carbohydr Polym. 2011;84:33–9.
30. Yu JS, Du KT, Fang Q, Gu Y, Mihardja SS, Sievers RE, Wu RC, Lee RJ. The use of human mesenchymal stem cells encapsulated in RGD modified alginate microspheres in the repair of myocardial infarction in the rat. Biomaterials. 2010;31:7012–20.
31. Davidovich-Pinhas M, Bianco-Peled H. Physical and structural characteristics of acrylated poly(ethylene glycol)–alginate conjugates. Acta Biomater. 2011;7:2817–25.
32. Wells LA, Sheardown H. Photosensitive controlled release with polyethylene glycol–anthracene modified alginate. Eur J Pharm Biopharm. 2011;79:304–13.
33. Hall KK, Gattas-Asfura KM, Stabler CL. Microencapsulation of islets within alginate/poly(ethylene glycol) gels cross-linked via Staudinger ligation. Acta Biomater. 2010;7:614–24.

34. Gattas-Asfura K, Stabler CL. Chemoselective cross-linking and functionalization of alginate via Staudinger ligation. Biomacromolecules. 2009;10:3122–9.
35. Mahou R, Wandrey C. Alginate-poly(ethylene glycol) hybrid microspheres with adjustable physical properties. Macromolecules. 2010;43:1371–8.
36. Mahou R. Thèse EPFL N. 2011, 5163.
37. Ceausoglu I, Hunkeler D. A new microencapsulation device for controlled membrane and capsule size distributions. J Microencapsulation. 2002;19:725–35.
38. Hisano N, Morikawa N, Iwata H, Ikada Y. Entrapment of islets into reversible disulfide hydrogels. J Biomed Mater Res. 1998;40:115–23.
39. Wu ZM, Zhang XG, Zheng C, Li CX, Zhang SM, Dong RN, Yu DM. Disulfide-crosslinked chitosan hydrogel for cell viability and controlled protein release. Eur J Pharm Sci. 2009;37:198–206.
40. Zelikin AN, Breheney K, Robert R, Tjipto E, Wark K. Cytotoxicity and internalization of polymer hydrogel capsules by mammalian cells. Biomacromolecules. 2010;11:2123–9.
41. Wandrey C, Espinosa D, Rehor A, Hunkeler D. Influence of alginate characteristics on the properties of multi-component microcapsules. J Microencapsulation. 2003;20:597–611.